CHAPTER 6
SUMMARY AND CONCLUSIONS
6.0 Summary

Hepatocellular Carcinoma (HCC) is the leading cause of cancer-related mortality worldwide due to its robust angiogenesis. There is an urgent need to find the angiogenic inhibitor with less toxicity as well as to develop the targeted drug delivery system. In the present study, firstly the plant based cytotoxic compounds (silymarin, silibinin, trans-chalcone and vitamin K1) were screened for their affinity with HCC receptors and later they were tested individually and in combinational treatment on HepG2 cell line. The maximum growth inhibition effect of each drug (sorafenib, silymarin and silibinin) individually was noted at its higher concentration (40 µM at 48 hour incubation). Combination of silibinin (20 µM) with sorafenib (20 µM) was enough to reduce cell viability up to 59% and 45% after 24 hour and 48 hours incubation respectively.

After in-vitro analysis, an animal model of HCC was developed and biochemical and histopathology confirmed the pathology. A significant elevated level of ALP activity was recorded. It was increased with HCC progression due to either tumor or cholestasis. Morphological analysis of liver tissue showed discoloration in the liver tissue after 30 days of DEN and 2AAF administration. After 60 day cirrhosis was observed in liver tissue. The liver was completely nodulated after 90 days. The nodules progressed into hepatic tumors on 120th day of model. The size of biggest tumor was observed to be 22 mm, which is the marker for tumor metastasis. Histopathological examinations showed distorted hepatic cells with vacuolization after 30 days of model progression. The number of vacuolated cells increased as disease progressed and showed immense vacuolated cells after 60th day. At 90th day clear nodulated structure was observed which culminated in visible adenoma after 120 days of dosing.
Sorafenib-silymarin (SF-SY) combination (1:1 ratio) and silibinin-PEG-sorafenib (SPS) conjugate were given (20 mg/Kg, 8 doses) to the diseased rats for evaluation of their efficacy as the angiogenic inhibitors. The biochemical analysis exhibited a significant reduction in the level of liver function enzymes with combinational treatment. Catalase activity was significantly improved after combinational treatment. While, Silibinin-PEG-sorafenib conjugate group exhibited marginal reduction in AST activity, the ALP level did not reduce. Histopathological analysis after Silibinin-PEG-sorafenib conjugate treatment showed reduction in tumor size. On the other hand, the treatment with sorafenib-silymarin combination exhibited improvement in liver function enzymes and the histopathological analysis revealed marginal significant reduction in tumors. This signifies that sorafenib-silymarin combinational treatment have better efficacy than silibinin-PEG-sorafenib conjugate.

Pullulan coated enteric tablets were prepared for sustained release of drugs in intestinal tract to enhance bioavailability of sorafenib-silibinin/silymarin. The tablets were prepared using sorafenib with silymarin and silibinin in 1:1 ratio. Maximum percent drug release was upto 98% for both drugs in sorafenib-silibinin (formulation 2), while it was 67% (sorafenib) and 90% (silymarin) for sorafenib-silymarin (formulation 1) combinational tablet. The biochemical analysis reveals that serum ALT, AST and ALP level were significantly decreased with both formulation treatment and formulation 2 (sorafenib–silibinin combination) showed maximum normalization in all the biochemical parameters as compared to the diseased group. Histopathological changes in the formulation 2 treated animals revealed a significant improvement in the hepatocytes and exhibited less disarrangement and degeneration of hepatocytes. It was noted that the adenomas were completely cured. This
improvement can be contributed to the synergistic effect of silibinin which has a strong antitumorogenic activity as well as it has the ability to decrease intracellular ROS levels in tumor bearing hepatocytes and antiangiogenic feature of both drugs.

Further the efficacy of the treatment by formulation 2 was evaluated using protein based identified Pharmacodynamic biomarkers. We had selected 11 proteins which were differentially expressed in disease with respect to control (details of the same are given in the result section). Out of the eleven proteins three proteins (APOC1, RasH and Protein kinase C eta) which are directly involved in angiogenic pathway were identified as Pharmacodynamic biomarkers to evaluate the efficacy of our treatment with formulation 2 at molecular level. It was observed that after the treatment with formulation 2, there was not only the biochemical and histopathological changes normalized and the three biomolecules were also identified biomarkers also recorded. The expression profiles of these identified proteins were at par with the control, which confirmed efficacy of sorafenib-silibinin formulation. The tablets have successfully inhibited the angiogenesis and tumorigenesis by regulating these proteins.

To elucidate mode of action for both drugs (sorafenib and silibinin) bioinformatics tools were used. The HCC signaling pathway was constructed using the identified proteins and the receptors were identified to see the docking scores of sorafenib-silibinin for understanding the mode of action of the formulation which has proved very effective in completely curing the HCC. The result of docking studies exhibited that sorafenib was inhibiting angiogenesis and cell proliferation through binding with VEGFR-β, B-Raf and Protein kinase B. Whereas, silibinin acted as antagonist for wnt/β-catenin pathway through inhibition of AXIN1 activation (binding with tankyrase) and β-catenin dephosphorylation (binding with PP2A).
The present study successfully develops a formulation of sorafenib-silibinin that is targeted and effective for the treatment of Hepatocellular carcinoma and has no side effects.

6.1 Conclusions
Based on the results of our studies the important conclusions drawn are summarized as under:

1. The cell line model was successfully developed by H$_2$O$_2$ in HepG2 cell lines.
2. The treatment with individual and combination drug suggests that sorafenib in combination with either silymarin or silibinin have synergistic effect and exhibits inhibition of tumorigenic hepatocytes.
3. A modified non-surgical method for induction of liver cancer in Wistar rats using a combination of DEN+2-AAF has been developed.
4. Validation of successful development of rodent model was achieved by biochemical and histopathological observations of the liver tissue.
5. On comparing the efficacy of sorafenib-PEG-silibinin conjugate and sorafenib-silibinin combination it was noted that the combination of sorafenib: silibinin proved to be more effective than the conjugate.
6. Pullulan coated tablets of sorafenib: silibinin exhibited enhanced bioavailability of drugs as well as pH specific sustained release in GI tract.
7. The sustained release tablets of drug combinations (sorafenib:silibinin) could decrease intracellular ROS levels and improve the activity of liver function enzymes.
8. Eleven differentially expressed proteins were identified by MALDI-TOF-MS which can be used as signature proteins to mark the HCC progression.

9. Three proteins (APOC1, RasH and Protein kinase C eta) have been identified as pharmacodynamic biomarker due to their presence in serum and tissue and their involvement in angiogenic pathway.

10. The evaluation of efficacy of tablets of drug combination at molecular level was confirmed by the evaluation of the expression by pharmacodynamics biomarker proteins reaching back to normal level after the treatment with pullulan coated tablets of sorafenib:silibinin.

11. The histopathology of (pullulan coated tablet having sorafenib:silibinin) treated animals confirmed the normalization of liver tissue and total elimination of tumors.

12. Bioinformatics tools successfully helped in the identification of molecular target for elucidation of the mode of action for sorafenib and silibinin.

13. The protein interaction analysis of 11 identified proteins helped in understanding the signaling in hepatocarcinogenesis and identification of HCC specific receptors.

14. Docking studies exhibited that sorafenib has ability to inhibit angiogenesis through binding with VEGFR-β, B-Raf and Protein kinase B. Whereas, silibinin acted as antagonist for wnt/β-catenin pathway through inhibition of AXIN1 activation (binding with tankyrase) and β-catenin dephosphorylation (binding with PP2A). Therefore, both compounds synergistically inhibited the angiogenesis in HCC and resulted in the cure of live cancer.